

APPLICATION INFORMATION

DNA Fragment Analysis

HIGHLY PRECISE DNA SIZING ON THE CEQ™ GENETIC ANALYSIS SYSTEM

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Capillary electrophoresis is rapidly supplanting conventional slab gel electrophoresis in moderate- to high-throughput laboratories that desire automated separations with a simple user interface. Capillary systems offer a level of automation and ease of use that cannot be achieved with slab gels. There is no ambiguity as to the identity of the sample in a capillary system, and the use of internal size standards allows a level of precision that far exceeds the visual comparison of adjacent lanes on a slab gel. Our data demonstrates that the CEQ™ Genetic Analysis System from Beckman Coulter precisely sized microsatellite amplification products in the size range of 200, 300, and 400, to a standard deviation of ≤ 0.27 bases, regardless of which capillary within an array, run, gel lot, or instrument was used to separate the sample.

Materials and Methods

Three different loci from the Weber panel were used in this study:

Locus	D18S858	D6S1040	D14S742
Marker Name	ATA23G05	GATA23F08	GATA74E02
Allele Size Range (nt)	193–208	257–285	395–415
Expected Alleles for Genomic DNA 1331-01	196, 196	277, 281	403, 403
Dye/Trace Color	D3/Green	D4/Blue	D2/Black

Primer sets were obtained from Research Genetics, Inc. (Huntsville, Alabama). The genomic DNA was CEPH Utah pedigree 1331, repository DNA sample NA07057A obtained from the Coriell Institute for Medical Research (Camden, NJ). Labeled primers were tested for quality by dilution to 30 fM in deionized formamide, and separated on the CEQ. Primer pairs were quantitated using a DU® 7500 UV Spectrophotometer (Beckman Coulter, Fullerton, CA.). Genomic DNA was quantitated on a FLUOstar-P Microplate Fluorometer (BMG Lab Technologies, Offenburg, Germany) using Pico Green (P-7581; Molecular Probes, Eugene, Oregon). Lambda DNA was used as a quantitative standard for this assay.

Individual amplification reactions were mixed as follows:

10X AmpliTaq Gold PCR Buffer	1.0 μ L
5X dNTPs (1.25 mM)	0.8 μ L
Primer Mix (2.5 mM each)	0.5 μ L
MgCl ₂ (25 mM)	0.6 μ L
DNA (20 ng/ μ L)	2.0 μ L
Enzyme (5U/ μ L)	0.05 μ L
Water	5.05 μ L
Total Reaction Volume	10.0 mL

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Capillary Electrophoresis

Cycling conditions were: 95°C—10 minutes, 1 cycle to activate the enzyme (AmpliTaq Gold,* Perkin Elmer, Branchburg, NJ), 95°C—1 minute, 57°C—1 minute, 72°C—1 minute, 40 cycles (MJ PTC 200 Thermocycler, MJ Research, Inc. Waltham, MA). The individual samples were then run on a CEQ™ Genetic Analysis System to determine the dilutions needed to balance the signal intensities of the size standards and amplification products. Mixtures large enough for 96 wells were prepared, and 40 μL of mixture was aliquoted into each well. For each sample well, the equivalent of 0.5 μL of the three PCR amplifications, 0.5 μL of the CEQ DNA Size Standard-400 (P/N 608098), and 39 μL of deionized formamide were added. Samples were run on a CEQ 2000 under the conditions given in Table 1.

Seven full plates were electrophoresed with two of three variables held constant (Table 2). The three variables were gel lot, array, and instrument. During the course of these experiments, several independent preparations of sample were done, as indicated in Table 3. In two cases, the same plate was used for a second injection.

Results and Discussion

The alleles generated in this experiment are illustrated in Figure 1. In all four cases, a +A artifact peak was generated, representing a different fraction of the overall locus-specific amplification product. The true allele, not the +A form of the allele, was

* AmpliTaq Gold is a registered trademark of Roche Molecular Systems, Inc.

Table 1. Frag-3 Run Method

Capillary Temperature	50°C
Denature Temperature and Time	90°C for 120 seconds
Injection Voltage and Time	2.0 kV for 30 seconds
Separation Voltage and Time	6.0 kV for 35 minutes

Table 2. Plates Run with Two of Three Variables Held Constant

Experiment	Gel	Array	Instrument
1	1	1	1
2	1	1	2
3	1	1	3
4	1	2	3
5	1	3	3
6	2	3	3
7	3	3	3

used in calculating the mean and standard deviation of the allele sizes in Table 4. Note that, in this table, two of the data sets are duplicated as indicated in the Experiment column.

The four peaks differed slightly from their expected sizes with the differences ranging from 0.1 to 1.7 bases. The differences were consistent across all experiments and appear to be independent of fragment length. Such variations are likely due to slight differences in composition of size standards,

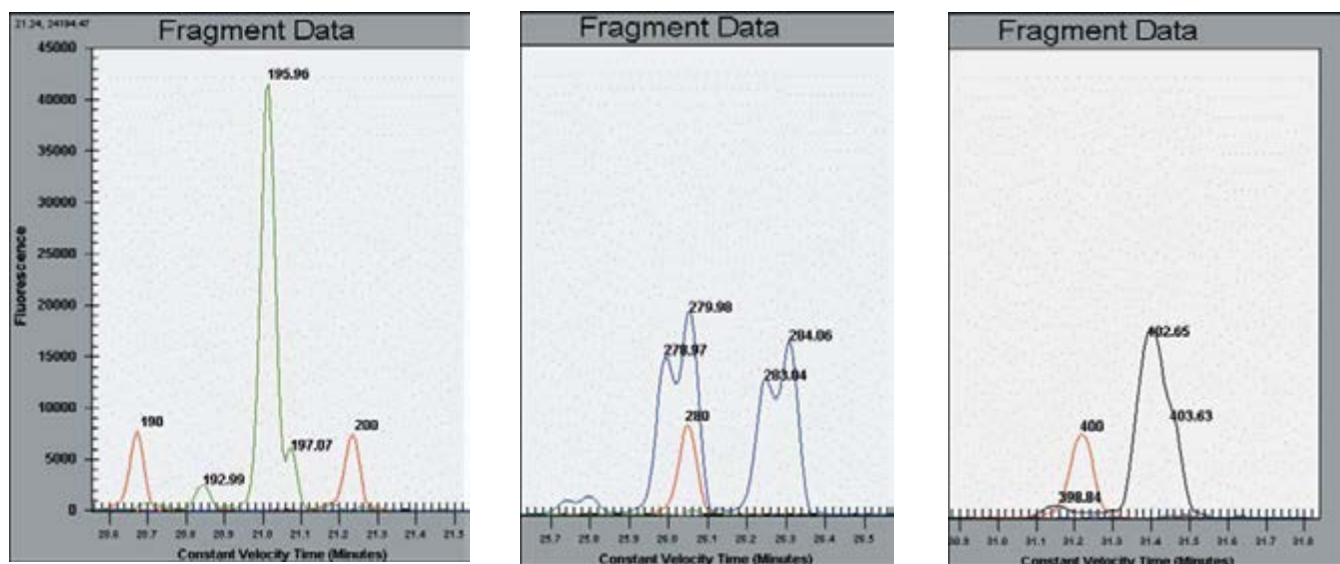


Figure 1. The four fragments used in the accuracy and precision tests. In each case, the first peak of the doublet was selected. The second peak was regarded as the +A artifact. The black peak includes the +A peak as shoulder on the main peak. The size standards are the peaks in red.

Table 3. Independent Amplifications and Preparation of Sample Mixtures for the Accuracy and Precision Tests

<i>Experiment</i>	<i>Amplification</i>	<i>Mixture</i>	<i>Injection</i>	<i>Operator</i>
1	1	1	1st	1
2	1	1	2nd	1
3	2	1	1st	2
4	2	2	1st	2
5	3	1	1st	2
6	3	2	1st	2
7	3	2	2nd	2

gel formulations, run temperatures, and other differences in the electrophoresis platforms. When comparing data obtained from different fragment sizing platforms, a series of known samples is often used to determine the corrections that must be applied in order to obtain the correct allele assignments. Due to the high level of precision demonstrated above, the size corrections determined at any locus on the CEQ™ alone should be constant as long as the fragments are run using the same temperature and voltage.

Within each of the seven plates examined, there was no noticeable drift of sizes from the first to the last row of a plate and no rows or capillaries that demonstrated unusual deviation from the mean sizes calculated from the fragment lists. Thus, samples appear to be stable within the time it takes to run an entire plate (approximately nine hours for 400 bases of separation). The capillary array that was used on three different instruments, consisting of more than one third of its expected useable lifetime, did not demonstrate a trend toward producing progressively higher or lower called sizes with increasing run numbers. Finally, the three different gel lots tested gave the same results, indicating a high level of reproducibility in the process used to prepare the Beckman Coulter polymer mixture. As expected, independent amplifications and preparations of the sample mixtures by different operators had no effect on the results. A second injection of sample was performed twice in the above experiments with no ill effects. While there is negligible sample depletion during each injection, some signal loss is expected due to formamide degradation, a process that is accelerated at high temperatures.

As a whole, the standard deviation observed with the 196-base fragment was less than half of the value obtained for the other three fragments. The reasons for this high level of precision are not known at this time. It is possible that the deviation from the mean fragment length is smaller due to the

short residence time in the capillary, allowing less time for diffusion. It is also possible that, under the run conditions used here, the population of 196-base fragments is more homogeneous in shape than the other three populations due to its particular sequence. Clearly, more examples would be required before any conclusions could be drawn.

In general, the ability to properly determine the correct number of repeat units in STR amplification products requires that a system be more precise than half the length of a repeat unit over a reasonable range of expected sizes. For example, to assign a tetranucleotide repeat allele to its proper size bin (range of observed sizes for a unique allele), a precision of better than two bases is required, and one-base precision is required for dinucleotide repeats. To be of general utility in large gene-mapping projects, however, a fragment sizing system must reproducibly determine fragment lengths of alleles containing imperfect repeats. Thus, better than 0.5-base precision is desirable since alleles containing repeat units of any size may, in rare cases, differ in overall length by only a single nucleotide. We demonstrated that, under a variety of circumstances likely to be encountered by CEQ users, the standard deviation about the mean is no greater than 0.26 bases.

Conclusions

Among the simple tandem repeat fragments tested, the CEQ Genetic Analysis System demonstrates outstanding reproducibility in calling fragment sizes. This level of precision would facilitate properly identifying alleles that differ by only one nucleotide in length up to at least 400 bases. The precision is achieved with consistency in the manufacturing of gels, the coating of capillaries, the uniformity of capillary temperature and run voltage, and the ability of the software to accurately identify peak centers.

Table 4. Observed Means and Standard Deviations from 96-Well Plates

<i>Experiment</i>		<i>Expected Allele> Size (bases)</i>	196	277	281	403
		Observed Sizes				
1	Instrument 1	Mean	196.16	278.53	282.62	402.45
		S.D.	0.04	0.12	0.10	0.11
2	Instrument 2	Mean	196.21	278.86	282.94	402.69
		S.D.	0.05	0.13	0.13	0.14
3	Instrument 3	Mean	196.19	278.40	282.48	402.28
		S.D.	0.03	0.13	0.13	0.09
	Across Instruments	Mean	196.19	278.59	282.68	402.47
		S.D.	0.05	0.24	0.24	0.21
3	Array 1	Mean	196.19	278.40	282.48	402.28
		S.D.	0.03	0.13	0.13	0.09
4	Array 2	Mean	196.14	278.67	282.74	402.49
		S.D.	0.05	0.13	0.12	0.11
5	Array 3	Mean	196.07	278.69	282.77	402.45
		S.D.	0.05	0.12	0.14	0.13
	Across Arrays	Mean	196.13	278.58	282.66	402.40
		S.D.	0.18	0.19	0.19	0.15
5	Gel 1	Mean	196.07	278.69	282.77	402.45
		S.D.	0.06	0.12	0.12	0.13
6	Gel 2	Mean	195.95	278.46	282.53	401.98
		S.D.	0.03	0.09	0.09	0.10
7	Gel 3	Mean	195.91	278.50	282.57	401.95
		S.D.	0.03	0.06	0.06	0.11
	Across Gels	Mean	195.98	278.54	282.62	402.13
		S.D.	0.08	0.15	0.15	0.27
	Across All Variables	Mean	196.10	278.57	282.65	402.33
		S.D.	0.12	0.20	0.20	0.26

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